Available online at www.sciencedirect.com

SCIENCE DIRECT

Biochimica et Biophysica Acta 1743 (2005) 215–222

BIOCHIMICA ET BIOPHYSICA ACTA BBA

http://www.elsevier.com/locate/bba

Regulation of cadmium-induced apoptosis by PKCδ in U937 human promonocytic cells

Begoña G. Miguel[a], M. Elena Rodriguez[a], Patricio Aller[b], Ana M. Martinez[a], Felicísima Mata[a,*]

[a]*Dept. Bioquímica y Biología Molecular I, Facultad de Biología, Universidad Complutense de Madrid, 28040 Madrid, Spain*
[b]*Centro de Investigaciones Biológicas, CSIC, 28040 Madrid, Spain*

Received 23 March 2004; received in revised form 29 July 2004; accepted 15 October 2004
Available online 28 October 2004

Abstract

Pulse treatment with cadmium chloride followed by recovery caused apoptosis in U937 human promonocytic cells. In addition, the treatment-induced PKCδ translocation from cytosol to membrane fraction, which was already detected at 30 min of treatment; and also caused PKCδ cleavage to give a 41-kDa fragment, which was detected at 3–6 h of recovery, concomitantly with the execution of apoptosis. All these effects were reduced by the PKCδ-specific inhibitor rottlerin. By contrast, rottlerin did not prevent the cadmium-provoked stimulation of the stress response (as measured by HSP70 expression), nor inhibited the generation of apoptosis by heat-shock, which failed to cause PKCδ translocation. Cadmium chloride rapidly induced $p38^{MAPK}$ activation, which was not affected by rottlerin. By contrast, the $p38^{MAPK}$ inhibitor SB203580 reduced PKCδ translocation and cleavage, indicating that $p38^{MAPK}$ activation precedes and regulates PKCδ activation. It is concluded that PKCδ mediates apoptosis induction by cadmium ions via early membrane translocation, and also possibly through late kinase proteolytic cleavage and phosphorylation on tyrosine residues.

Keywords: Cadmium; Apoptosis; PKCδ translocation; PKCδ cleavage; $p38^{MAPK}$ activation; U937 cell

1. Introduction

Cadmium is a widespread environmental contaminant with mutagenic, carcinogenic and teratogenic effects [1]. Exposure to this heavy metal causes both acute as well as chronic poisoning, due to its persistent accumulation in animal tissues [2]. It has been indicated that cadmium may induce apoptotic and necrotic cell death, depending on the used cell type, the used concentration, and the experimental design [3–9]. In particular, we demonstrated in preceding publications that treatment with cadmium chloride induced apoptosis in U937 human promonocytic cells, but the mode of death was switched to necrosis if the treatment was applied under glutathione depletion conditions [7,10]. In addition, the efficacy of apoptosis was conditioned by the occurrence of the stress ("heat-shock") response, which, as manifested by HSF1 ("heat-shock factor 1") activation and HSP70 expression, is also elicited by cadmium ions in this and other myeloid cell types [11,12]. The phenotypic characteristics of both apoptosis and necrosis are in general well defined, but the mechanisms that regulate cell death are less known.

One of the most relevant aspects in the regulation of apoptosis is the signalling by protein kinases. Serine/threonine kinases represent a broad category of kinases, which include, among others, the mitogen-activated protein kinases (MAPKs) and the calcium-dependent protein kinases (PKCs) [13]. The PKC family itself includes several isoforms, classified as (i) calcium-dependent, or classical PKCs (cPKCs: α, β1, β2, and γ), which are activated by diacylglicerol (DAG) or 12-*O*-tetradecanoylphorbol-13-acetate (TPA); (ii) calcium-independent, or novel PKCs (nPKCs: δ, ϵ, η, and θ), which are activated by DAG or TPA; and (iii) atypical PKCs

* Corresponding author. Tel.: +34 913 944 156; fax: +34 913 944 672.
E-mail address: fmata@bbm1.ucm.es (F. Mata).

0167-4889/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamcr.2004.10.011

(aPKCs: σ; ζ and λ), which are calcium-independent and are not activated by DAG or TPA [14]. These isozymes present important differences in their enzymatic properties, cell type expression, substrate selectivity, and subcellular localization [15]. Different mechanisms of PKC activation have been described, including translocation to plasma membrane, nucleus or mitochondria, proteolysis, and tyrosine phosphorylation [16–18].

The capacity of cadmium ions to cause PKC activation, and the involvement of PCK on cadmium-induced apoptosis, was earlier studied with noncoincident results. For instance, while cadmium ions activated PKC, which in turn seemed to mediate apoptosis induction in lung epithelial cells [19], the heavy metal did not activate PKC in rat mesangial cells [20]; and cadmium-induced apoptosis was apparently independent of PCK activation in rat glioma cells [9]. In the present work, we analyze the possible role of PKCδ on apoptosis induction by the cadmium chloride in U937 promonocytic cells. The results indicate that cadmium ions cause an early PKCδ translocation to membrane fraction, and a late PKCδ cleavage and phosphorylation on tyrosine residues, which may be important for apoptosis. In addition, PKCδ activation seems to be mediated by the prior activation of $p38^{MAPK}$.

2. Materials and methods

2.1. Materials

All components for cell culture were obtained from Invitrogen, Inc. (Carlsbad, CA, USA). Cadmium chloride, TPA, genistein, propidium iodide (PI), and mouse anti-chicken β-tubulin monoclonal antibody were obtained from Sigma (Madrid, Spain). 4,6-Diamino-2-phenylindole (DAPI) was obtained from Serva (Heidelberg, Germany); and rottlerin, SB203580 and mouse anti-phosphotyrosine monoclonal antibody (PY20), from Calbiochem (Darmstadt, Germany). All anti-PKC antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-human $p38^{MAPK}$ and rabbit anti-human phospho-$p38^{MAPK}$ (Thr180/Tyr182) polyclonal antibodies were from Cell Signalling Technology (Beverly, MA, USA); mouse anti-human HSP70 monoclonal antibody (clone C92F34-5) from StressGen Biotechnologies Corp. (Victoria, Canada); and goat anti-rabbit and anti-mouse peroxidase-conjugated antibodies from DAKO Diagnostics, S.A. (Barcelona, Spain).

2.2. Cells and treatments

Human U937 myeloid leukemia cells [21] were grown in RPMI 1640 medium supplemented with 10% heat-inactivated calf serum and 0.2% sodium bicarbonate and antibiotics in a humidified 5% CO_2 atmosphere at 37 °C. Cadmium chloride was prepared in distilled water at 50 mM just before application. TPA, rottlerin, SB203580 and genistein were dissolved in dimethyl sulfoxide at 1.6, 10, 13.2 and 30 mM, respectively, and stored at −20 °C. For cadmium treatments, the cells were incubated for the desired time periods with 200 μM cadmium chloride; and for heat shock, the cells were placed in a bath at 42 °C for the desired time periods. Then, the cells were washed twice with prewarmed RPMI medium, and allowed to recover under standard culture conditions in the absence of the drug. For PKC translocation studies, cells were used immediately after cadmium ion treatment. As control, cells were subjected to the same manipulations as treated cells, at 37 °C and without the drug.

2.3. Determination of apoptosis and necrosis

Distinctive characteristics of apoptotic cells were the presence of chromatin condensation/fragmentation and the acquisition of sub-G_1 DNA content. To analyze changes in chromatin structure, the cells were collected by centrifugation, washed with phosphate-buffered saline (PBS), resuspended in PBS, and mounted on glass slides. After fixation in 70% (v/v) ethanol, the cells were stained for 20 min at room temperature with 1 μg/ml DAPI and examined by fluorescence microscopy. To measure sub-G_1 DNA content, cells were collected by centrifugation and incubated for 30 min in PBS containing 0.5 mg/ml RNase A. After the addition of 50 μg/ml PI and permeabilization with 0.1% (w/v) Nonidet P-40, the cells were analyzed by flow cytometry. Within the experimental time periods used here necrotic cells did not exhibit sub-G_1 PI incorporation. On the other hand, cells undergoing typical "primary" necrosis exhibited marked swelling and loss of plasma membrane integrity, as evidenced by permeability to trypan blue (by microscopy) or by massive influx of PI in nonpermeabilized cells (by flow cytometry).

2.4. Cell lysis and subcellular fractionation

The whole procedure was carried out at 4 °C, and all used media were supplemented with 1% (v/v) 2-mercaptoethanol, 0.1 mM sodium metavanadate, 20 mM sodium fluoride, 10 mg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride, immediately before use. After washing three time times with cold PBS, the cells were resuspended in a buffer containing 20 mM Tris–HCl, pH 7.5, 5 mM $MgSO_4$, and 0.5 mM EGTA, and lysed with 50 strokes of a Dounce homogenizer using a B-type pestle. Complete cell lysis was confirmed by microscopy. When the total lysate had to be directly used for immunoblot assays, the lysis was completed by sonication. Otherwise, the lysate was centrifuged at 800×*g* for 10 min. The pellet was used to isolate nuclei. The supernatant was used to isolate cytosolic and membrane fractions, as described below.

The nuclei were obtained by the method of Fields et al. [22]. Briefly, the 800×*g* pellet was resuspended in STM buffer (50 mM Tris–HCl, pH 7.4, 250 mM sucrose and 5 mM $MgSO_4$) and capped over a buffer containing 2.1 M sucrose, 50 mM Tris–HCl, pH 7.4, 5 mM $MgSO_4$, and 1% v/v 2-mercaptoethanol. Nuclei were successively pelleted at 70,000×*g* for 60 min in a SW 28 rotor. Purified nuclei were resuspended in STM buffer and incubated for 60 min with DNase I and RNase A (100 μg/ml each). Nuclease-treated nuclei were sedimented at 800×*g* for 10 min and resuspended in 50 mM Tris–HCl, pH 7.4, at 5×10^8 nuclei/ml.

The 800×*g* supernatant was centrifuged at 100,000×*g* for 60 min, and the supernatant collected was used as the cytosolic fraction as described previously [16]. The pellet was resuspended in buffer containing 50 mM Tris–HCl, pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA and 1% nonidet P-40, shaked for 60 min, and centrifuged at 100,000×*g* for 60 min. The resulting supernatant was used as the membrane fraction. Marker enzymes were used in conjunction with electron microscopy to ensure the purity of fraction as described previously [23].

2.5. Immunoblot and immunoprecipitation analysis

Immunoblot analysis was basically carried out as previously described [18]. Equal amounts of protein (100 μg, as determined by the Bradford's method [24]) from the total extract or from each individual fraction were electrophoresed on 8% SDS-polyacrylamide gels and then transferred to nitrocellulose membranes. After blocking the nonspecific sites with TBS-T solution [0.1% (v/v) Tween 20 in 20 mM Tris base, 137 mM NaCl, pH 7.6] containing 1% (w/v) BSA for 3 h at room temperature, the membranes were incubated with the primary antibody overnight at 4 °C. The membranes were then washed three times (10 min each) in TBS-T solution, and incubated for 2 h at room temperature with horseradish peroxidase-linked secondary antibody (1/1000 dilution in TBS-T solution containing 4% skimmed milk). After washing four times (10 min each) in TBS-T solution, the bound antibody was detected by enhanced chemiluminescence method according to the manufacturer's (Amersham, Uppsala, Sweden) instructions. The scanned images of autoradiograms were quantified using the Sigmagel image program.

Immunoprecipitation of PKCδ was performed as described previously [25]. Briefly, after treatment, U-937 cells (10^7) were washed with phosphate-buffered saline and then harvested in 600 μl of immunoprecipitation buffer containing 25 mM HEPES, pH 7.2, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 50 mM KCl, 1% Triton X-100, 0.1% SDS, 10 mM NaF, 10 mM $Na_4P_2O_7$, 1.2 mM Na_3VO_4, 2.5 μg/ml aprotinin, 2.5 μg/ml pepstatin, 2.5 μg/ml leupeptin and 1 mM phenylmethanesulfonyl fluoride. The cell suspension was sonicated and then centrifuged at 120,000×*g* for 30 min. The supernatant was incubated with 4 μg anti-PKCδ antibody and 20 μl of protein A agarose beads at 4 °C overnight. The immunoprecipitates were washed twice with lysis buffer and then analyzed by Western blotting with anti-phophotyrosine antibody (PY20) as described above. As control, the membranes were routinely reproved using an anti-β tubulin antibody.

3. Results

3.1. Apoptosis induction

Fig. 1 shows the capacity of cadmium chloride to cause cell death in U937 cells. The experimental design (pulse-treatment followed by recovery), as well as the cadmium chloride concentration (200 μM), were directly adopted from our earlier studies [10,26]. It was observed that the treatment provoked a significant apoptosis (about 50% after 120 min of treatment and 6 h recovery), as manifested by the appearance of cells with condensed/fragmented chromatin (Fig. 1) and the acquisition of sub-G_1 DNA content (results not shown). The frequency of apoptosis depended on the length of both the treatment (Fig. 1A) and recovery (Fig. 1B) periods. Other

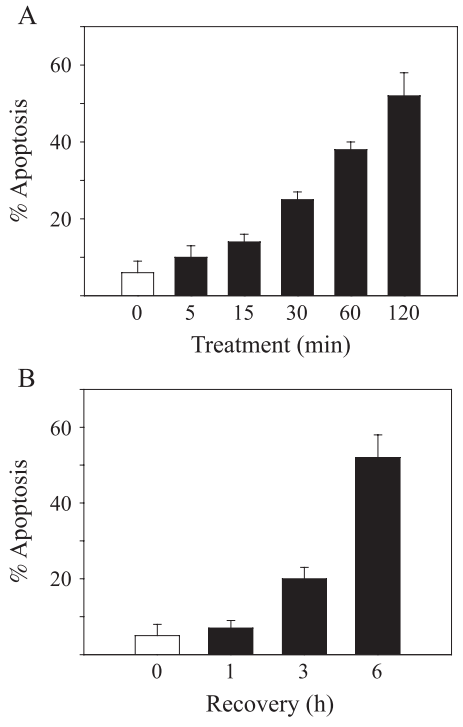

Fig. 1. Induction of apoptosis by cadmium chloride in U937 cells. The histograms show the frequency of apoptotic cells, as determined by chromatin condensation/fragmentation, in U937 cell cultures treated for the indicated time periods with 200 μM cadmium chloride, and then allowed to recover for 6 h in drug-free medium (A); and in cells treated for 120 min with 200 μM cadmium chloride, and then allowed to recover for the indicated time periods (B). The values represent the mean±SD of three experiments.

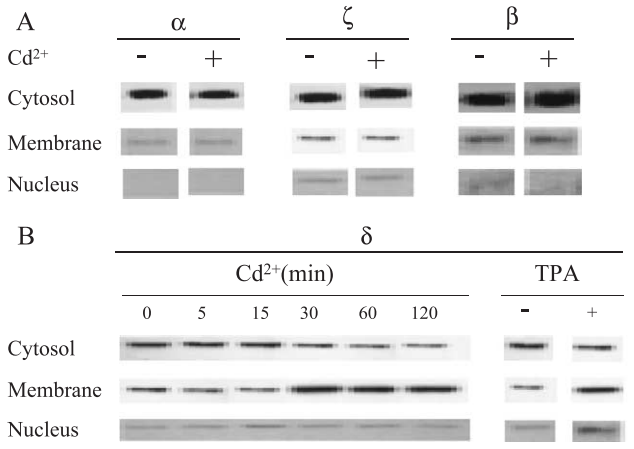

Fig. 2. Intracellular redistribution of PKC isoforms after cadmium chloride treatment. The figure shows the relative amounts of PKC α, ζ, β and δ in cytosolic, membrane and nuclear fractions, as determined by subcellular fractionation and immunoblot, using appropriate antibodies. (A) Levels of the indicated PKC isoforms in untreated cells (−) and cells treated for 120 min with 200 μM cadmium chloride (+). (B) Levels of PKCδ in cells treated for the indicated time periods with cadmium chloride, and in cells incubated for 60 min in the absence (−) and or the presence (+) of 250 nM TPA. The blots are representative of one of three determinations with similar results. The frequency of apoptotic cells was determined at 6 h of recovery as in Fig. 1. The values represent the mean±SD of three experiments.

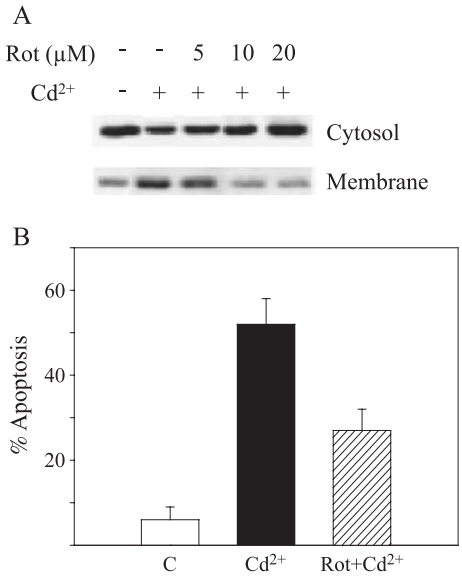

Fig. 3. Effects of rottlerin on cadmium-induced PKCδ translocation and apoptosis. U937 cell cultures were treated for 120 min with cadmium chloride, with or without the indicated concentrations of rottlerin, which was added 30 min before cadmium chloride. (A) Translocation of PKCδ to the membrane fraction, at the end of the 120-min treatment period. (B) Frequency of apoptotic cells in untreated cultures, in cultures treated with cadmium chloride alone (Cd^{2+}), and in cultures treated with cadmium chloride plus 10 μM rottlerin ($Rot+Cd^{2+}$), measured at 6 h of recovery in drug-free medium. The data are the mean±SD of three experiments.

apoptotic markers, as well as caspase activation and the effect of caspase inhibitors, were already examined in preceding works [7,26], and hence are omitted here. Under the used conditions, the frequency of necrotic cells remained negligible (approximately 4%, as in untreated cultures).

3.2. PKCδ translocation, cleavage, phosphorylation on tyrosine residues, and effect of PKC inhibitor

One of the possible manifestations of PKC activation is kinase redistribution between the cytosol and other intracellular compartments [16,18,27]. For these reason, immunoblot assays using specific antibodies against different PKC isoforms were carried out to determine possible changes in intracellular PCK localization in cadmium-treated cells. Among the different PKC isoforms, only PKCα, βI, δ, and ζ were readily detectable in U937 cells (Fig. 2, and results not shown). It was observed that cadmium ions elicited a significant translocation of PKCδ from cytosol to the membrane fraction, in a similar manner as TPA (here used as a control), while the redistribution of the other isoforms remained unaltered upon cadmium chloride treatment (Fig. 2A). PKCδ translocation in response to cadmium chloride was already detected at 30 min of treatment, hence preceding the execution of apoptosis, and was maintained for at least 120 min (Fig. 2B). On the other hand, cadmium ions did not cause significant translocation of PKC isoforms to nuclei fraction, which, at least in the case of PKCδ, is provoked by TPA (Fig. 2B).

To examine the possible cause–effect relationship between PKCδ translocation to membrane fraction and apoptosis induction, experiments were carried out using rottlerin, which is known to preferentially inhibit this PKC isoform [28]. It was observed that rottlerin prevented PKCδ translocation, reaching the maximum effect at the concentration of 10 μM (Fig. 3A). At this concentration, the inhibitor partially prevented the execution of apoptosis (Fig. 3B), indicating that PKCδ activation/translocation in fact mediates death induction by cadmium ions. At the assayed conditions, treatment with rottlerin alone did not

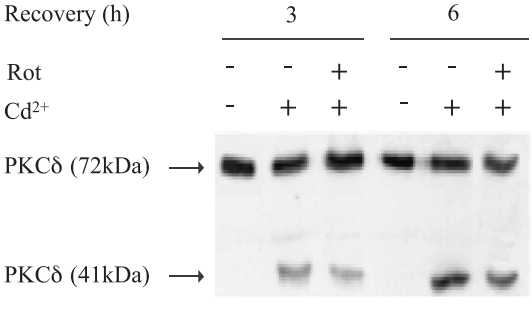

Fig. 4. PKCδ cleavage. The figure shows the relative amount of the entire PKCδ isoform (72 kDa) and its derived catalytic fragment (41 kDa) in untreated cells and cells treated for 120 min with cadmium chloride, with and without 10 μM rottlerin, and allowed to recover in drug-free medium for the indicated time periods. The blot is representative of one of three experiments with similar results.

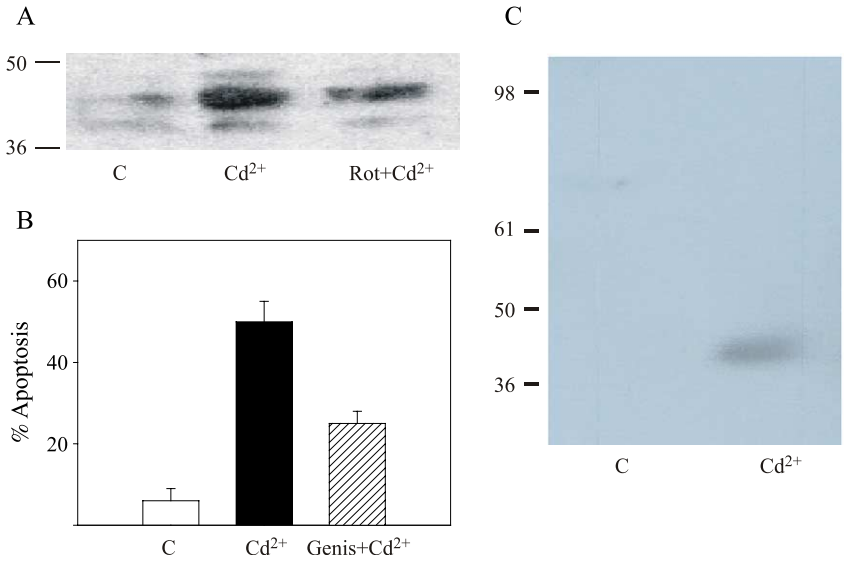

Fig. 5. PKCδ tyrosine phosphorylation. (A) The figure shows the relative level tyrosine phosphorylation of a protein of approximately 41 kDa in untreated cells and cells treated for 2 h with cadmium chloride, either alone (Cd^{2+}) or in combination with 10 μM rottlerin (Rot+Cd^{2+}), as determined by immunoblot using an anti-phosphotyrosine antibody (PY20). The experiment was repeated twice with similar results. (B) The histogram shows the frequency of apoptotic cells in untreated cultures and cultures treated for 120 min with cadmium chloride, alone (Cd^{2+}) or in combination with the 30 μM genistein (Genis+Cd^{2+}). Genistein was added 15 min before cadmium chloride and maintained during the cadmium treatment and recovery period. The values represent the mean±SD of three experiments. (C) Tyrosine phosphorylation of PKCδ immunoprecipitated with the anti-PKCδ antibody and analyzed by immunoblotting with PY20. The experiment was repeated twice with similar results. All determinations were carried out at 6 h of recovery.

cause PKCδ intracellular redistribution nor apoptosis (results not shown).

Cells treated with apoptosis-inducing agents undergo proteolytic PKCδ cleavage, to give a catalytically active fragment that may contribute to apoptosis [29]. For this reason, we wanted to measure PKCδ cleavage in response to cadmium chloride treatment, with and without rottlerin. Immunoblot assays indicated that cadmium ions elicited the appearance of the characteristic apoptosis-associated 41-kDa fragment, the amount of which was reduced by rottlerin (Fig. 4). This fragment was detected at 3–6 h of recovery after treatment, concomitantly with the execution of apoptosis (see Fig. 1B) and the activation of caspase-3 [7]. Treatment with rottlerin alone did not cause the appearance of PKCδ-derived 41-kDa fragment (results not shown). This is congruent with the common consideration of PKCδ cleavage as a late event, mediated by executioner caspase-3 activity [17]. No apparent cleavage was detected in α, βI, and ζ isoforms under these conditions of treatment (results not shown). Of note, immunoblot assays using an anti-phosphotyrosine antibody revealed a prominent band of approximately 41 kDa, the intensity of which was increased during the recovery period, and which was reduced by rottlerin (Fig. 5A). Moreover, we observed that the specific tyrosine kinase inhibitor genistein decreased the frequency of cadmium-induced apoptosis (Fig. 5B). At the assayed conditions, apoptosis was not affected by the treatment with genistein alone (data not shown). This findings may be congruent with earlier observations indicating that tyrosine phosphorylation of PKCδ influences its catalytic and pro-apoptotic activity [17,30]. To further analyze the effect of cadmium ions on tyrosine phosphorylation of PKCδ, extracts were obtained from cells at 6 h of recovery after cadmium chloride treatment,

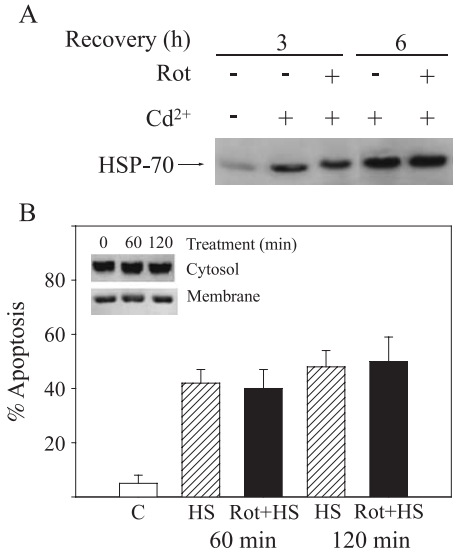

Fig. 6. Effect of rottlerin on cadmium-induced HSP70 expression and heat-shock-induced apoptosis. (A) The figure shows the relative level of the stress-inducible HSP70 in untreated cells and cells treated for 120 min with cadmium chloride, in the absence and presence of rottlerin, as determined by immunoblot at the indicated times of recovery. The experiment was repeated twice with similar results. (B) The histogram shows the frequency of apoptotic cells in cultures heated at 42 °C for 60 or 120 min in the absence (HS) or presence (Rot+HS) of 10 μM rottlerin, and allowed to recover for 6 h. The values represent the mean±SD of three experiments. The inset shows the amount of PKCδ in the cytosolic and membrane fractions, at the indicated times of heating.

PKCδ was immunoprecipitated with an anti-PKCδ antibody, and the membrane was blotted with anti-phosphotyrosine antibody (PY20). As illustrated in Fig. 5C, the PKCδ-derived 41-kDa fragment resulted phosphorylated by the treatment, in agreement with the results showed in Fig. 5A.

It has been indicated that rottlerin inhibits cadmium uptake in some cell types [31]. For this reason, we found it of interest to analyze the action of the kinase inhibitor on another well-known effect of cadmium, namely the induction of the stress ("heat-shock") response [12,26]. We observed that rottlerin did not affect the cadmium-elicited increase in HSP70 expression (Fig. 6A), ruling out the possibility that the decreased toxicity could be a trivial consequence of transport inhibition or other mechanism of cadmium inactivation [31]. Rottlerin alone did not affect HSP70 expression (results not shown). In addition, to exclude the possibility of a general, nonspecific inhibition of the apoptotic machinery by rottlerin, unrelated to its action as kinase inhibitor, U937 cells were subjected to heat-shock treatment. It was observed that heat shock did not cause PKCδ translocation, and in agreement with this rottlerin did not affect the heat-provoked apoptosis (Fig. 6B). Taken together, these results indicate that the attenuation of cadmium-provoked apoptosis by rottlerin was a highly specific effect.

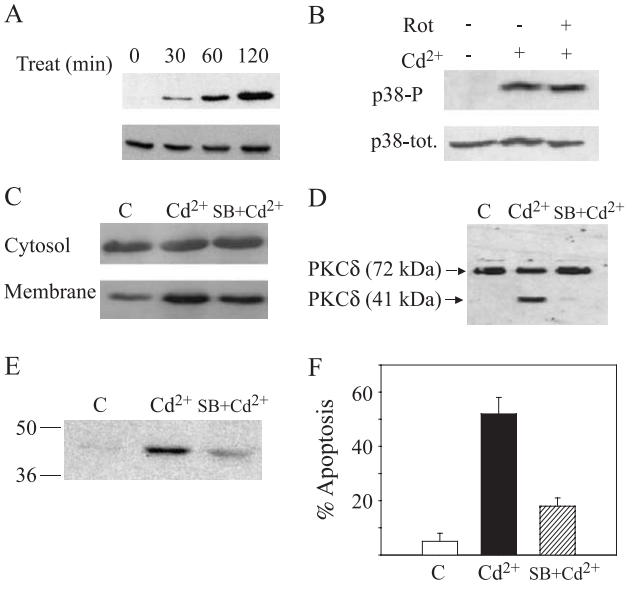

Fig. 7. Relationships between $p38^{MAPK}$ activation, PKCδ activation, and apoptosis, in cadmium-treated cells. Panels A and B show the relative levels of total (p38-tot) and phosphorylated (p38-P) $p38^{MAPK}$ at the indicated times of treatment with cadmium chloride (A), or at 120 min of treatment with cadmium chloride in the absence or presence of rottlerin (B). Panels C–D show the amount of PKCδ in cytosolic and membrane fractions (C), PKCδ cleavage (D), tyrosine phosphorylation level of the 41-kDa fragment (E), and frequency of apoptotic cells (F), in untreated cultures and cultures treated for 120 min with cadmium chloride, either in the absence (Cd^{2+}) or the presence ($SB+Cd^{2+}$) of 10 μM SB203580, and allowed to recover for 6 h. SB203580 was applied 1 h before cadmium chloride. All blots are representative of one of at least two determinations with similar results. The values in F represent the mean±SD of three experiments.

3.3. $p38^{MAPK}$ activation

We have previously reported that cadmium ions cause a rapid activation of $p38^{MAPK}$, which mediate apoptosis induction in U937 cells [7]. In agreement with this, in the present experiments, we detected an increase in $p38^{MAPK}$ phosphorylation at 30 min of cadmium chloride treatment (Fig. 7A), which coincides with the timing of PKCδ translocation (see Fig. 2B). In order to study the possible cause–effect relationship between PKCδ and $p38^{MAPK}$ activation, experiments were carried out using rottlerin and SB203580, a specific inhibitor of $p38^{MAPK}$. It was observed that rottlerin did not affect the basal level of $p38^{MAPK}$ phosphorylation (results not shown) nor the increase in phosphorylation elicited by cadmium ions (Fig. 7B). By contrast, SB203580 decreased the amount of PKCδ detected in membrane fraction (Fig. 7C), indicating that in our experimental model $p38^{MAPK}$ precedes and regulates the early PKCδ translocation. In addition, SB203580 greatly reduced PKCδ cleavage (Fig. 7D), reduced tyrosine phosphorylation of the 41-kDa fragment (Fig. 7E), and attenuated apoptosis (Fig. 7F). SB203580 alone did not affect the PKCδ distribution/cleavage nor apoptosis (results not shown).

4. Discussion

PKC activities have been demonstrated to regulate important cellular functions, such as proliferation, differentiation, and apoptosis [32,33]. Nevertheless, the effect PKC activation on these processes is not always consistent. Thus, the observation that TPA and other general PKC activators may either prevent [34–38] or potentiate [36,37,39,40] cell death indicates that PKC may exert both anti- and pro-apoptotic effects, depending on the cell model and experimental conditions. When specific PKC isoforms are considered, PKCζ and λ activities have been shown to protect against apoptosis, and PKCα and δ activities to mediate apoptosis induction, in multiple cell types [38–40]. The involvement of PKCδ in apoptosis is currently examined using rottlerin, a drug that preferentially inhibits this particular isotype, but the results must be considered with caution because of the possible collateral effects of pharmacological inhibitors. For instance, Blass et al. [30] reported that rottlerin attenuated the cytotoxic action of cadmium ions in CHO cells, but this effect was attributed to the capacity of the drug to inhibit metal uptake in this particular cell line. In the present work, we observed that rottlerin attenuates the cadmium-provoked apoptosis in human U937 promonocytic cells. Although metal uptake was not directly examined, the finding that rottlerin did not affect the stress reaction (as measured by HSP70 expression) indicates that the protective

effect is not a trivial consequence of transport inhibition or other mode of cadmium ion inactivation (e.g., direct drug–metal interaction).

Several possible mechanisms for PKCδ activation have been described, including kinase translocation from cytosol to other cellular compartments, and kinase cleavage by caspase-3 (or a related enzyme) to give a catalytically active fragment [17]. In the present work, we observed that cadmium chloride elicit a rapid translocation of PKCδ from cytosol to membrane (particulate) fraction, preceding all manifestations of apoptosis. The importance of translocation for cadmium-provoked apoptosis was proved by the facts that rottlerin inhibited both the cadmium-provoked kinase translocation and cell death, and that the toxicity of heat shock, which did not cause kinase translocation, was not affected by rottlerin. On the other hand, cadmium ions apparently failed to cause PKCδ increase in the nuclear fraction, which nevertheless was identified as possible target of translocation by other agents [27,41]. In addition, we observed a late PKCδ cleavage to give the typical apoptosis-associated 41-kDa fragment, the amount of which was also reduced by rottlerin. Nevertheless, due to the strict temporal coincidence of this event with the expression of apoptotic markers, we may not clearly discriminate whether PKCδ cleavage is a mere consequence of apoptosis or it also plays a regulatory role, as proposed by some authors [20]. Finally, we observed that cadmium ions induced phosphorylation on tyrosine residues in the 41-kDa fragment obtained by proteolytic PKCδ cleavage, and that genistein reduced the cadmium-provoked apoptosis. This may be of interest in the framework of the present investigation, since tyrosine phosphorylation has been described as a mechanism that modulates (either enhancing or decreasing) the catalytic and pro-apoptotic activity of PKCδ.

Although clearly distinguishable, there are multiple evidences of cross-talk between PKC and MAPK pathways. We were interested in investigating the possible relationship between PCKδ and $p38^{MAPK}$ because this latter kinase seems to be particularly important for the regulation of apoptosis in U937 [7] and other cell models [42]. Actually, several proofs of a link between these kinase activities in relation to apoptosis have been offered, with the frequent observation that PKCδ precedes and regulates $p38^{MAPK}$ [43]. Our results confirm the existence of cause–effect relationship between these kinases in cadmium-treated promonocytic cells, but in our experiments $p38^{MAPK}$ seems to be upstream PKCδ activation. Whether this is a particularity of the experimental model (cell type and/or apoptosis inducer), or it may acquire a more general value, remains to be determined.

Acknowledgements

We thank Dr. M.C. Calcerrada and Mr. D. Martin for expert technical assistance. This work was partially supported by Grant SAF-2001-1219 from the Plan Nacional de Investigacion Cientifica, Desarrollo e Investigación Tecnológica, Ministerio de Ciencia y Tecnología; by Grant 01/0946 from the Fondo de Investigación Sanitaria, Ministerio de Sanidad y Consumo; and by Grant 08.3/0011.3/2001 from the Comunidad Autónoma de Madrid, Spain.

References

[1] E. Gebhart, T.G. Rossman, in: E. Merian (Ed.), Metals and their Compounds in the Environment, VCH Publishers Inc., New York, 1991, pp. 617–640.
[2] A.F. Morselt, Environmental pollutants and diseases. A cell biological approach using chronic cadmium exposure in the animal model as a paradigm case, Toxicology 70 (1991) 1–132.
[3] B.A. Hart, C.H. Lee, G.S. Shukla, A. Shukla, M. Osier, J.D. Eneman, J.F. Chiu, Characterization of cadmium-induced apoptosis in rat lung epithelial cells: evidence for the participation of oxidant stress, Toxicology 133 (1999) 43–58.
[4] T. Zhou, G. Zhou, W. Song, N. Eguchi, W. Lu, E. Lundin, T. Jin, G. Nordberg, Cadmium-induced apoptosis and changes in expression of p53, c-jun and MT-I genes in testes and ventral prostate of rats, Toxicology 142 (1999) 1–13.
[5] S. Somji, D.A. Sens, S.H. Garrett, M.A. Sens, J.H. Todd, Heat shock protein 27 expression in human proximal tubule cells exposed to lethal and sublethal concentrations of $CdCl_2$, Environ. Health Perspect. 107 (1999) 545–552.
[6] S.S. Habeebu, J. Liu, C.D. Klaassen, Cadmium-induced apoptosis in mouse liver, Toxicol. Appl. Pharmacol. 149 (1998) 203–209.
[7] A. Galán, M.L. García-Bermejo, A. Troyano, N.E. Vilaboa, E. Blas, M.G. Kazanietz, P. Aller, Stimulation of p38 mitogen-activates protein kinase is an early regulatory event for the cadmium-induced apoptosis in human promonocytic cells, J. Biol. Chem. 275 (2000) 11418–11424.
[8] M. Li, T. Kondo, Q.L. Zhao, F.J. Li, K. Tanabe, Y. Arai, Z.C. Zhous, Z.M. Kasuya, Apoptosis induced by cadmium in human lymphoma U937 cell through Ca^{2+}-calpain and caspase-mitochondria-dependent pathways, J. Biol. Chem. 275 (2000) 39702–39709.
[9] W. Watjen, M. Cox, M. Biagioli, D. Beyersmann, Cadmium-induced apoptosis in C6 glioma cells: mediation by caspase 9-activation, Biometals 15 (2002) 15–25.
[10] A. Galán, L. García-Bermejo, A. Troyano, N.E. Vilaboa, C. Fernández, E. Blas, P. Aller, The role of intracellular oxidation in death induction (apoptosis and necrosis) in human promonocytic cells treated with stress inducers (cadmium, heat X-rays), Eur. J. Cell Biol. 80 (2001) 312–320.
[11] S. Lindquist, E.A. Craig, The heat-shock proteins, Annu. Rev. Genet. 22 (1988) 631–677.
[12] N.E. Vilaboa, C. Calle, C. Pérez, E. de Blas, L. García-Bermejo, P. Aller, cAMP increasing agents prevent the stimulation of heat-shock protein 70 (HSP70) gene expression by cadmium chloride in human myeloid cell lines, J. Cell. Sci. 108 (1995) 2877–2883.
[13] T.G. Cross, D. Scheel-Toellner, N.V. Henriquez, E. Deacon, M. Salmon, J.M. Lord, Serine/threonine protein kinases and apoptosis, Exp. Cell Res. 256 (2000) 34–41.
[14] H. Mellor, P.J. Parker, The extend protein kinase C superfamily, Biochem. J. 332 (1998) 281–292.
[15] Y. Nishizuka, The molecular heterogeneity of protein kinase C and its implications for cellular regulation, Nature 334 (1988) 661–665.
[16] B.G. Miguel, M.C. Calcerrada, F. Mata, P. Aller, R. Clemente, R.E. Catalán, A.M. Martínez, Differential redistribution of protein kinase C isoforms by cyclic AMP in HL60 cells, Biochem. Biophys. Res. Commun. 274 (2000) 596–602.

[17] C. Brodie, P.M. Blumberg, Regulation of cell apoptosis by protein kinase C δ, Apoptosis 8 (2003) 19–27.
[18] M.C. Calcerrada, B.G. Miguel, L. Martín, R.E. Catalán, A.M. Martínez, Involvement of phosphatidylinositol 3-kinase in nuclear translocation of protein kinase C ζ induced by C2-ceramide in rat hepatocytes, FEBS Lett. 514 (2002) 361–365.
[19] R.D. Watkin, T. Nawrot, R.J. Potts, B.A. Hart, Mechanisms regulating the cadmium-mediated suppression of Sp1 transcription factor activity in alveolar epithelial cells, Toxicology 184 (2–3) (2003) 157–178.
[20] Z. Wang, D.M. Templeton, Induction of c-fos proto-oncogene in mesangial cells by cadmium, J. Biol. Chem. 273 (1998) 73–79.
[21] C. Sundström, K. Nilsson, Establishment and characterization of a human histiocytic lymphoma cell line (U-937), Int. J. Cancer 17 (1976) 565–577.
[22] A.P. Fields, G.R. Pettit, W.S. May, Phosphorylation of lamin B at the nuclear membrane by activated protein kinase C, J. Biol. Chem. 263 (1988) 8253–8260.
[23] M.C. Calcerrada, B.G. Miguel, R.E. Catalán, A.M. Martínez, Sphingosylphosphorylcholine increases calcium concentration in isolated brain nuclei, Neurosci. Res. 33 (1999) 229–232.
[24] M.M. Bradford, A rapid and sensitive method for the quantization of microgram quantities of protein utilizing the principle of protein–dye binding, Anal. Biochem. 72 (1976) 248–254.
[25] J.A. Tapia, L.J. García-Marín, T. Jensen, Cholecystokinin-stimulated protein kinase C-delta kinase activation, tyrosine phosphorylation, and translocation are mediated by Src tyrosine kinases in pancreatic acinar cells, J. Biol. Chem. 278 (2003) 35220–35230.
[26] A. Galan, A. Troyano, N.E. Vilaboa, C. Fernández, E. de Blas, P. Aller, Modulation of the stress response during apoptosis and necrosis induction in cadmium-treated U-937 human promonocytic cells, Biochim. Biophys. Acta 14716 (2000) 1–9.
[27] D. Scheel-Toellner, D. Pilling, A.N. Akbar, Inhibition of T cell apoptosis by IFN-β rapidly reverses nuclear translocation of protein kinase C-delta, Eur. J. Immunol. 8 (1999) 2603–2612.
[28] M. Gschwendt, H.J. Muller, K. Kielbassa, R. Zang, W. Kisttein, G. Rincke, F. Marks, Rottlerin, a novel protein kinase inhibitor, Biochem. Biophys. Res. Commun. 199 (1994) 93–98.
[29] Y. Emoto, Y. Manome, G. Meinhardt, H. Kisaki, S. Kharbanda, M. Robertson, T. Ghayur, W.W. Wong, R. Kamen, R. Weichselbaum, D. Kufe, Proteolytic activation of protein kinase C δ by an ICE-like protease in apoptotic cells, EMBO J. 14 (1995) 6148–6156.
[30] M. Blass, I. Kronfeld, G. Kazamirsky, P.M. Blumberg, C. Brodie, Tyrosine phosphorylation of protein kinase C δ is essential for its apoptotic effect in response to etoposide, Mol. Cell. Biol. 22 (2002) 182–195.
[31] J.H. Chen, C.H. Huang, L.Y. Lin, Rottlerin stimulates metallothionein gene expression but inhibits metal transport in Chinese hamster ovary cells, Toxicol. Appl. Pharmacol. 177 (3) (2001) 256–263.
[32] Y. Nishizuka, The molecular heterogeneity of protein kinase C and its implications for cellular regulation, Nature 334 (1988) 661–665.
[33] Y. Nishizuka, Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C, Science 258 (1992) 607–614.
[34] J. Lotem, E.J. Cragoe Jr., L. Sachs, Rescue from programmed cell death in leukemic and normal myeloid cells, Blood 78 (1991) 953–960.
[35] C. Widmann, S. Gibson, G.L. Johnson, Caspase-dependent cleavage of signaling proteins during apoptosis. A turn-off mechanism for anti-apoptotic signals, J. Biol. Chem. 273 (1998) 7141–7147.
[36] R.A. Franklin, J.A. McCubrey, Kinases: positive and negative regulators of apoptosis, Leukemia 14 (2000) 2019–2034.
[37] J. Pongracz, W. Tuffley, G.D. Johnson, E.M. Deacon, D. Burnett, R.A. Stockley, J.M. Lord, Changes in protein kinase C isoenzyme expression associated with apoptosis in U937 myelomonocytic cells, Exp. Cell Res. 218 (1995) 430–438.
[38] E. Berra, M.T. Díaz-Meco, I. Dominguez, M.M. Municio, L. Sanz, J. Lozano, R.S. Chapkin, J. Moscat, Protein kinase C ζ isoform is critical for mitogenic signal transduction, Cell 74 (1993) 555–563.
[39] X. Zhao, J.E. Gschwend, C.T. Powell, R.G. Foster, K.C. Day, Retinoblastoma protein-dependent growth signal conflict and caspase activity are required for protein kinase C-signaled apoptosis of prostate epithelial cells, J. Biol. Chem. 272 (1997) 22751–22757.
[40] M.F. Denning, Y. Wang, B.J. Nickoloff, T. Wrone-Smith, Protein kinase C δ is activated by caspase-dependent proteolysis during ultraviolet radiation-induced apoptosis of human keratinocytes, J. Biol. Chem. 273 (1998) 29995–30002.
[41] R. Datta, D. Banash, H. Kojima, R. Talanian, E. Alnemri, W. Wong, D. Kufe, Activation of the CPP32 protease in apoptosis induced by 1-beta-D arabinofuranosyl-cytosine and other DNA-damaging agents, Blood 88 (1996) 1936–1943.
[42] J. Raingeaud, S. Gupta, J.S. Rogers, M. Dickens, J. Han, R.J. Ulevitch, R.J. Davis, Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine, J. Biol. Chem. 270 (1995) 7420–7426.
[43] M.C. Heidkamp, A.L. Bayer, J.L. Martin, A.M. Samarel, Differential activation of mitogen-activated protein kinase cascades and apoptosis by protein kinase C epsilon and delta in neonatal rat ventricular myocytes, Circ. Res. 89 (10) (2001 (Nov. 9)) 882–890.